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# Two Short-Term Toxicity Tests for the Calanoid Copepod 'Eurytemora herdmani' Using a Complex Effluent

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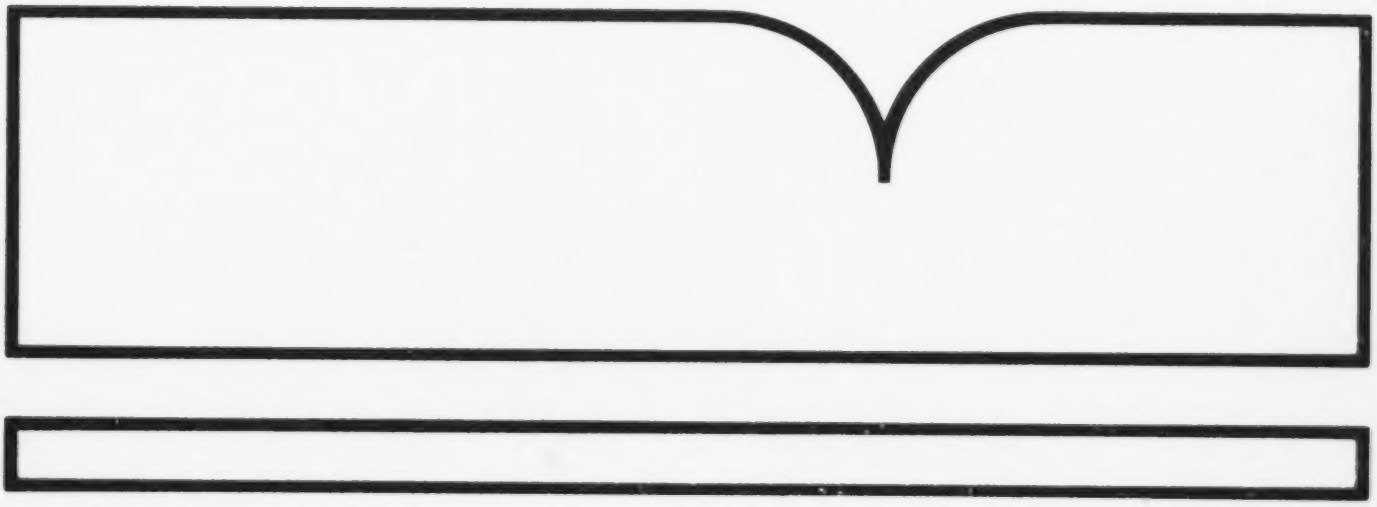

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# Two Short-Term Toxicity Tests for the Calanoid Copepod *Eurytemora herdmani* Using a Complex Effluent[1]

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**Abstract.** The test designs and methodologies for two short-term static renewal tests, a 96-hr lethality test and a 5-day reproductive test, are described and statistically evaluated. The tests were developed specifically for use in the assessment of the toxicity of mixed effluents to copepods. Two tests using the calanoid copepod, *Eurytemora herdmani*, and one effluent yielded comparable results. The results indicate that mortality is a fairly sensitive measure of toxicant effect and is more easily quantified than the reproductive endpoint. The test designs allowed for the detection of a 7.8-22% difference in adult mortality between the control and any effluent treatment with a power of approximately 0.98. A 70% difference between the control and any other treatment was necessary to detect a difference in the chosen reproductive endpoint at a power of 0.98.

Recently, interest has increased in estimating some of the potential biological effects resulting from the discharge of complex effluents into the marine environment. A suite of toxicity tests is currently under development for the Permits Division of the U.S. Environmental Protection Agency Office of Water at the U.S. EPA Environmental Research Laboratory, Narragansett, RI, for the routine assessment of the toxicity of these materials. The study is part of a larger program to develop inexpensive short term toxicity test methodologies with some predictive capability for use, in part, as discharge permit requirements. Two objectives have been to examine more sensitive alternatives to the relatively hardy species that have been traditionally used in marine toxicity testing (*Cyprinodon, Fundulus, Palaemonetes, Mercenaria*), and to pursue endpoints other than mortality.

The purpose is to develop and evaluate an experimental design and methodology for determining the toxicity of complex effluents to copepods. Practical considerations determined by the Agency led to the imposition of a number of constraints on the test design. These constraints were on the test duration, level of effort, transportability, and need for the design and methodology to be applicable to a variety of copepod species and effluents. The test needed to be seven days or less in duration and could not require more than an average of two hr of effort per day to complete. It needed to be relatively sensitive with methodologically easily measured variables. Finally, a reproductive endpoint was desired.

This study compares the results from a newly developed reproductive test with results obtained by an existing accepted toxicity test method, a 96-hr adult lethality test, using a single effluent. The test methodologies were derived from recently developed *Eurytemora* sp. methodologies for heavy metal (Marcy *et al.* unpublished) and sewage sludge toxicity tests (Miller *et al.* 1984).

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## Materials and Methods

The calanoid copepod, *Eurytemora herdmani* (Thompson and Scott), was used in the development of the laboratory tests. *Eurytemora herdmani* is a neritic calanoid copepod found along eastern North America between Labrador and Narragansett Bay, RI, (Wilson 1932; Carter 1965). A breeding laboratory population of this copepod was established at the U.S. Environmental Protection Agency, Environmental Research Laboratory, Narragansett, RI, in June, 1982 with approximately 100 females sorted from a Narragansett Bay plankton tow. The females were acclimated to 15°C, 25–30 ppt salinity Pettaquamscutt River (RI) water. Young from these females were used to establish the laboratory population which has been in continuous culture since its initiation. All *E. herdmani* tests were performed on newly matured adults from this population. Approximate generation time for this animal is 32 days at 15°C and 30 ppt salinity (George 1983).

All copepod life stages were fed a mixed algal diet of *Rhodomonas baltica*, *Skeletonema costatum*, and *Pavlova lutheri* combined in approximately equal amounts by volume. The phytoplankton were cultured in 100 ml lots in a modified f/2 medium (Guillard and Ryther 1962). Modifications to the algal medium were made to the trace metal mix: only the prescribed concentrations of EDTA, iron, zinc, and manganese were added. No other buffers or chelators were included. Silicates were added at half strength. Algae were grown at 20°C under 24 hr fluorescent illumination for the first seven days and were then placed in 15°C, 12L:12D fluorescent illumination. Only phytoplankton between seven and fourteen days post innoculation were fed to the copepods.

The *E. herdmani* were reared as cohorts *i.e.*, nauplii that had hatched within 7 days or less of each other were kept together throughout their lives. All animals were reared in 11.4 cm Carolina® culture dishes containing 150 ml Pettaquamscutt River water at approximate densities of 300 nauplii, 200–250 copepodids, or 150 adults/container. Stock cultures were reared at 10°C and 25–30 ppt salinity under 12L:12D fluorescent illumination. The animals were transferred weekly to clean 0.45 μm filtered Pettaquamscutt River water by either gentle pipetting or by gently sieving through a 210 μm Nitex® screen.

The stock copepods were fed different densities of algae depending on lifestage. Nauplii received the algal mix at approximately 0.25 ml algal mix/100 ml seawater, copepodids received 0.75 ml algal mix/100 ml seawater, and adults received 0.55 ml algal mix/100 ml seawater. These amounts were varied somewhat depending upon animal densities. All stock animals were fed once daily, 6 days/week.

The effluent selected for this study was obtained from Circuit-Wise Inc., New Haven, Connecticut, a metal finishing plant manufacturing electronic circuit boards. Circuit-Wise Inc. is monitored under NPEDS Permit No. CT0022420 for cyanide, aluminum, copper, iron, lead, nickel, tin, benzene, 1,1,1-trichloroethane, and trichloroethylene. A composite sample of the Circuit-Wise Inc. effluent, collected over a one-week period in August, 1984, was used for the copepod toxicity tests. No chemical analyses were performed on this sample.

Due to the relatively low toxicity and highly variable nature of the effluent, a wide range of concentrations was selected. For the reproductive test, a high concentration of 10% effluent was used. The lower concentrations were 3.20%, 1.00%, 0.32%, and 0.10% effluent and a natural seawater control. The 96-hr lethality test (using adult copepods) used concentrations of 10.0%, 5.00%, 2.50%, 1.25%, 0.625% effluent and a natural seawater control. The effluent preparations were mixed independently (not serially) on a percentage basis (see Marcy 1985 for preparation details).

The test animals were acclimated to the experimental conditions (15°C, 20 ‰ salinity) over a 72 hr period. Seventy-two hr before the test initiation, approximately 160 females and 160 males were isolated from the stock cultures and were placed in 25 ‰ salinity Pettaquamscutt River water at 10°C. Water at salinities less than 30 ‰ was made by diluting 30 ‰ water with deionized water. Forty-eight hr prior to test initiation, test animals were gently transferred by pipet to 20 ‰ salinity water. Twenty-four hr before test initiation, the containers with the test animals were placed in a 15°C (±2°C) controlled temperature box, where they remained until the toxicity test began 24 hr later. Water temperatures equilibrated in approximately 45 min during the acclimation period. The copepods were fed daily during this period.

### *Experimental Design*

Two static renewal tests were performed: 1) a 96-hr lethality test (with adult organisms), and 2) a 5-day reproductive test. A duration of five days was chosen for the reproductive test to allow the control animals to reproduce at least twice during the test.

Each treatment initially consisted of four, 60 ml replicates containing five females and five males each for a total of 40 animals in each concentration. Males and females were randomly assigned to test containers using the method described by Marcy (1985).

The animals were not fed during the 96-hr lethality test. Animals in the 5-day reproductive test were fed daily at the rate of 0.2 ml algal diet (described above) per 60 ml replicate container on the first day and 0.16 ml (= 80% of 0.2 ml of the algal diet) on all subsequent days.

Test containers were kept tightly covered and the medium was not aerated throughout the tests to minimize loss of any volatiles present in the effluent. Eighty-three percent (50 ml) of the test medium was replaced daily from each replicate with an equivalent amount of freshly mixed test solution. In order to save time in performing renewals, a siphoning device (Marcy 1985) was used to simultaneously siphon all four replicates of each test concentration. At the termination of the reproductive test, all larvae (alive plus recoverable dead) were either counted and recorded, or were preserved in 10% seawater formalin for later enumeration. Adult mortality was also evaluated and recorded at the time of test termination.

The toxic effects resulting from the complex chemical composition of effluents can make evaluation of copepod death difficult. Occasionally, a temporary anaesthetic effect has been observed with some toxic materials (Miller *et al.* 1984). Experience has shown that animals not responding to prodding (a generally accepted criterion for death (ASTM 1980)), were frequently observed actively swimming the following day. We therefore used the following criteria for determining adult copepod death. Any one of these criteria was considered sufficient to signify death:

1) Animals that were decaying were dead,
2) Animals that were totally opaque, either white, pink, or orange were dead,

[3] Mention of brand names does not imply endorsement by the U.S. Environmental Protection Agency.

**Table 1.** Some power function estimates and their associated probabilities for detecting the specified differences between means, as determined for the two toxicity tests performed on *Eurytemora herdmani*

| Test | Variable | d[a] | d*[a] | power |
|---|---|---|---|---|
| 96-hr Lethality Test | Male mortality | 0.25 | 8.8% | 0.98 |
| | Female mortality | 0.20 | 10.2% | 0.99 |
| | Combined mortality | 0.18 | 7.8% | 0.98 |
| 5-day Reproductive Test | Male mortality | 0.25 | 11.3% | 0.98 |
| | Female mortality | 0.35 | 22.0% | 0.98 |
| | Combined adult mortality | 0.30 | 17.6% | 0.99 |
| | Number of young recovered | 1.25 | 70.0% | 0.98 |

[a] d is the estimated difference in arcsine square root units, for mortality, or natural logarithm units, for reproduction; d* is the approximate percent difference between the control and any other mean that can be detected at the designated power (one-tailed test)

3) Animals showing limp appendages that swayed freely in light water movement were dead.
4) When observed from the dorsal side, animals that showed no gut movement for 60 sec in the 2nd to 4th metasomal area were considered dead.

### *Physical Parameters*

Temperature was maintained constant (±0.5°C) throughout the two studies by placing the test containers in a controlled temperature water bath. Temperature was recorded continuously by chart recorder (Dickson®) for each test. Salinity was maintained at 20 ppt (±2 ppt) and was measured at each water renewal (every 24 hr) using a refractometer (American Optical®). Adjustment to 20‰ salinity was accomplished by diluting 30‰ salinity Narragansett Bay water with deionized water. Dissolved oxygen concentrations were recorded once during each test after a 24 hr period using an oxygen meter (L.G. Nester Co.®, Model 8500 portable dissolved oxygen/BOD meter). Concentrations were at saturation for both tests. (Dissolved oxygen concentrations are required to be at a minimum of 60% of saturation for a valid test (ASTM 1980).)

### *Statistical Analysis*

Means and standard deviations were computed for all dependent variables at each test concentration and control. An arcsine square root transformation was performed on the mortality data, and a logarithmic (ln x) transformation was performed on the reproduction results to normalize the data for ANOVA. Each dependent variable (male, female, and combined male and female mortality; number of recoverable young) was analyzed by ANOVA (SAS Institute 1982a,1982b) to obtain mean square error values. In cases where significant differences were indicated by ANOVA ($P < 0.05$), *a posteriori* pairwise comparisons were made between each concentration and the control using a one-tailed Dunnett's Test ($\alpha = 0.05$)(Steel and Torrie 1960). Using the Dunnett's Test results, geometric means were computed for each dependent variable as the square root of the product of the highest no effect and the lowest effect concentrations.

LC50 values were determined by the binomial method and, where possible, by the moving average method (Thompson 1947) for male, female, and combined male and female mortalities at the termination of both experiments. Since the observed control mortalities were 10% and 17.5%, the data were not adjusted for control mortality using Abbott's correction. Software for the LC50 analyses was developed by C. Stephan (U.S. EPA, Environmental Research Laboratory, Duluth).

Estimates of the power function for each dependent variable were determined from the computed mean square errors (Snedecor and Cochran 1967)(Table 1). The power of the test is the probability of detecting a specified difference between the control mean and any other mean. These estimates are one way to appraise the sensitivity of the chosen experimental design to detect treatment vs control differences with the given mean and variance estimates and sample size. For example, a power of 0.98 indicates that a treatment vs control control difference is detectable with a 98% probability.

Finally, the natural logarithm of the male to female mortality ratio was computed for each replicate and was tested using the ANOVA to determine the presence or absence of significantly different mortality resonses to the effluent based upon the sex of the animal.

## Results

Tables 2 and 3 report means and standard deviations for the two tests with the Circuit-Wise Inc. effluent. Total adult control mortality was 10% in the 96-hr lethality test and 17.5% in the 5-day reproductive test. Control mortalities of these magnitudes are considered high by ASTM (1980) guidelines and will be discussed in a later section. Variability between replicates ranged from small to large and therefore the untransformed treatment variances showed considerable heterogeneity.

The geometric means of the highest no-effect and lowest-effect concentrations and the LC50 values determined from the mortality data were reasonably close in both the 96-hr lethality test and the 5-day reproductive test; the largest difference between any two values was slightly over a factor of two (Tables 2, 3, and 4). The male mortality results from the 96-hr, 0.625% effluent concentration were omitted from this computation because of unusually high male mortality. LC50 values for the male

**Table 2.** Results of the 96-hr lethality test with adult *Eurytemora herdmani* exposed to Circuit-Wise Inc.[a] effluent[b]

| Concentration (% effluent) | Male mortality | Female mortality | Combined mortality |
|---|---|---|---|
| control | 0.3(0.5) | 0.8(0.5) | 1.0(0.8) |
| 0.625 | 1.8(1.0)* | 0.0(0.0) | 1.8(1.0) |
| 1.25 | 0.8(1.0) | 0.0(0.0) | 0.8(1.0) |
| 2.50 | 1.0(0.0) | 0.5(0.6) | 1.5(0.6) |
| 5.00 | 1.8(1.0)* | 1.0(0.0) | 2.8(1.0)* |
| 10.0 | 5.0(0.0)* | 4.3(0.5)* | 9.3(0.5)* |

* Indicates a value significantly different ($P < 0.05$) from the control (Dunnett's test)

[a] A metal finishing plant, New Haven, CT

[b] Mortality data are summarized as means of four replicates per treatment (± standard deviations), in numbers of animals. Ten animals (five females and five males) were exposed per replicate. The geometric means of the highest no effect and lowest effect concentrations (% effluent) are: male mortality, 3.54; female mortality, 7.07; combined mortality, 3.54

*E. herdmani* could not be determined by the moving average method in the 5-day reproductive test.

Reproduction appears to be a more sensitive indicator of effluent toxicity in this study than does adult mortality. The geometric mean of the highest no effect and lowest effect concentrations from the reproductive results was approximately 32% of that of the geometric mean determined from combined adult mortality in the same test. The magnitude of this difference (32%) may be a function of the effluent dilutions used (3.2).

The results of the ANOVA performed on the male to female mortality ratios showed no significant difference ($P > 0.05$) between male and female mortality responses to the effluent in the reproductive study. Significant differences ($P = 0.004$) occurred in the male to female ratio in the 96-hr lethality test, which may be in large part due to the high male mortality in the 0.625% effluent treatment.

## Discussion

The experimental design of five effluent concentrations plus a control appears adequate for the required purpose of an easily performed test intended to give approximate results. The prescribed experimental concentration intervals in the reproductive test are too wide (intervals are on a 3.2 scale) to be consistently useful in LC50 determination by any method more rigorous than the binomial test. This precludes computation of tight confidence intervals. Geometric means of the highest no-effect and lowest-effect concentrations, however, may easily be determined to give estimates of effluent toxicity.

The variances computed from the raw data are largely unequal, thereby violating the assumption of homogeneity of variances for performing an ANOVA. It was, therefore, necessary to perform appropriate transformations on all of the raw test results. A large compromise between simplicity of test design and a consequent increase in the heterogeneity of variances was made in the 5-day reproductive test. The chosen reproductive endpoint was used only for expediency. Inherent in this endpoint are numerous sub-endpoints all contributing to the variability of the result, *e.g.*, adult, particularly female, mortality; variations in egg production rates and brood sizes; and larval death and decay rates. The results of the reproductive test are also not directly comparable to endpoints used in any population growth models or to most other reproductive endpoints reported in the literature. As toxicological work of this nature tends to be labor intensive with *Eurytemora*, the experimental design used here represents the maximum in sample size and replication that can be reasonably handled under the given experimental constraints.

A one-tailed Dunnett's test was chosen over tests such as Duncan's Multiple Range test or Tukey's Studentized Range test, because it allows only treatment vs control comparisons rather than all treatment comparisons and is considered to be more powerful for this purpose (ASTM Guidelines). The one-sided alternative renders the test insensitive to those concentrations showing enhanced survivals or reproduction over the controls and increases the power to detect differences in test concentrations resulting in depressed survival or reproduction. The power function estimates reported in Table 1 indicate that the chosen design is adequate to detect differences of 22% or more between means in adult mortality, a fairly sensitive test.

The reproductive test is far less powerful for detecting treatment vs control differences, *i.e.*, a difference between two means must be greater in the reproductive test (at least 70% of the control) to be detectable. In all probability, this is due to the compromises in choosing the particular reproductive endpoint discussed earlier. Since power is a function of both the variance and replication, assuming no changes in treatment means or significance level, increasing the number of replicates per concentration will increase the power. However, replication must be increased to 10 replicates per concentration to give a power of 0.98 to detect even a

**Table 3.** Results of the 5-day reproductive test with *Eurytemora herdmani* exposed to Circuit-Wise Inc.[a] effluent[b]

| Concentration (% effluent) | Male mortality | Female mortality | Combined mortality | # Young recovered |
|---|---|---|---|---|
| control | 0.5(0.6) | 1.3(1.9) | 1.8(2.2) | 57.8(13.9) |
| 0.10 | 0.0(0.0) | 0.5(1.0) | 0.5(1.0) | 85.5(12.4) |
| 0.32 | 0.8(1.0) | 0.3(0.5) | 1.0(1.4) | 76.0(26.1) |
| 1.0 | 0.0(0.0) | 0.3(0.5) | 0.3(0.5) | 53.3(27.6) |
| 3.2 | 0.3(0.5) | 0.3(0.5) | 0.5(1.0) | 16.8 (6.4)* |
| 10.0 | 5.0(0.0)* | 5.0(0.0)* | 10.0(0.0)* | 2.0 (2.2)* |

* Indicates a value significantly different ($P < 0.05$) from the control (Dunnett's test)

[a] A metal finishing plant, New Haven, CT

[b] Mortality and reproductive results are summarized as means of four replicates per treatment (± standard deviations), in numbers of animals. Ten animals (five females and five males) were exposed per replicate. The geometric means of the highest no effect and lowest effect concentrations (% effluent) are: male mortality, 5.66; female mortality, 5.66; combined male plus female mortality, 5.66; number of recovered young, 1.79

**Table 4.** LC50 values for the 96-hr lethality test and the 5-day reproductive test, with *Eurytemora herdmani*, exposed to Circuit-Wise Inc.[a] effluent

| | | LC50 (95% CI) (% effluent)[b] | |
|---|---|---|---|
| Test | Variable | Binomial | Moving average |
| 96-hr Lethality Test | Males | 5.65(0.00–10.0) | 4.30(3.42–5.61) |
| | Females | 6.85(5.00–10.0) | 6.71(5.32–9.34) |
| | Combined males and females | 6.23(5.00–10.0) | 5.73(4.93–6.79) |
| 5-day Reproductive Test (120-hr LC50) | Males | 5.2(3.2–10) | — |
| | Females | 5.2(3.2–10) | 4.9(3.8–6.7) |
| | Combined males and females | 5.2(3.2–10) | 4.9(4.1–5.9) |

[a] A metal finishing plant, New Haven, CT

[b] LC50 values were determined by binomial and moving average methods

55% difference between the control mean and an experimental concentration in the reproductive test. Furthermore, although the reproductive test appears to be somewhat more sensitive in detecting effluent effects, the geometric mean of the highest no-effect and the lowest-effect concentrations, based on the reproductive test, is only slightly over a factor of three of the geometric mean based on adult mortality. Replicate tests on the same effluent must be conducted to determine whether these geometric means are actually statistically different.

Since male and female copepods are kept together in the test containers, the experimental design violates the requirement of independence needed to make direct statistical comparison of male and female responses. Analysis of the male to female mortality ratios showed that although male and female mortalities may have occurred at different rates throughout both tests, no change in the male to female ratio could be attributed to exposure to the effluent in the reproductive test. Significant differences were detected, however, between male and female mortality ratios in the 96-hr lethality test. The differential responses observed in the two tests may be due to the high male mortality obtained in the 0.625 effluent concentration. More investigation into this phenomenon is warranted.

This and other short term copepod toxicity studies (Miller *et al.* 1984) have shown somewhat higher control mortalities than are generally acceptable by ASTM (1980). Previous work with sewage sludge has shown that increasing the numbers of animals and/or control replication does not appreciably reduce mean control mortality. High control mortalities may be a result of the acclimation regime, although tests with sewage sludge following a much simpler acclimation procedure also yielded relatively high control mortalities (Miller *et al.* 1984). Although further research into this question is indicated, some mortality could be attributed to natural phenomena, to be expected in a species whose lifespan is relatively short (a maximum of 60–70 days for females at 15°C (George 1983)).

The static renewal system used in these tests was

a simple system requiring little test maintenance. It should be noted, however, that this design is suitable only for effluents with low particle loading and are light enough in pigmentation to allow viewing of the animals. The entire experimental system is easily transportable to a distant site of less than 2–3 days transportation time, if the animals and their algal food are protected from fluctuations in environmental conditions, especially temperature. Maintaining a laboratory population of *E. herdmani* can be the most costly aspect of these studies as culture is labor intensive and requires specialized skills. This latter problem could be alleviated by obtaining experimental animals directly from the plankton. Of course, this may limit animal availability and the researcher has no control over, and little information on, the animals' history. Choice of another copepod that is simpler to rear (*e.g.* the harpacticoid *Nitocra spinipes*, utilized extensively for toxicological work in Europe (Bengtsson 1978, 1981; Linden *et al.* 1979; Renberg *et al.* 1980; Bengtsson and Bergstrom 1982), may reduce costs. The test methods described here are applicable to a variety of other herbivorous or omnivorous copepod species with little or no modification other than diet for the reproductive test. Laboratory culture of many species, however, is difficult, and plankton-caught animals may be simpler to use.

A final note must be made regarding the small but significant amount of EDTA that is a constituent in the f/2 algal food medium used in the reproductive test. EDTA is a chelator and can reduce metals toxicity in tests where it is used. F/2 medium is the medium of choice for copepod work as it contains the least amount of possible toxicants as additives and promotes good algal growth. As it is necessary to feed the copepods in order for them to reproduce, the addition of a very small (ppb range) amount of EDTA is unavoidable in the reproductive test.

## Summary

Two short-term toxicity tests for mixed effluents are performed using the calanoid copepod *Eurytemora herdmani*. One test, a 96-hr lethality test, measures male and female mortality over a range of effluent concentrations and is based on standard ASTM (1980) methods. The other test evaluates reproduction and adult mortality after a five day period. A comparison of the two tests indicates that mortality is a relatively sensitive measure of toxicant effect. Furthermore, there is no appreciable difference between mortality observed in the 96-hr and the 5-day tests. The male or female mortality ratio varied with effluent concentration in the 96-hr lethality test possibly due to an outlier in the 0.625% effluent concentration. There was no difference in the mortality response over the effluent concentration range based on male to female mortality ratios in the 5-day reproductive test.

The particular reproductive endpoint (number of recoverable young) for the 5-day test was selected solely for simplicity of measurement. Heterogeneity of variances resulted in a reduction in the power of the statistical analyses to detect more than relatively large differences between means. Although the results of the reproductive test gave a geometric mean of the highest no-effect and lowest-effect concentrations lower than that computed from the mortality results, the statistical significance of this difference cannot be determined from this study.

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